

06/12/00
12846 U.S.P.T.O.

06-13-00 A

Please type a plus sign (+) inside this box →

Approved for use through 09/30/2000. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	MBX 012 CON
First Inventor or Application Identifier	David P. Martin
Title	METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS
Express Mail Label No.	EL 381 208 545 US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. Specification [Total Pages 29]
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. Drawing(s) (35 U.S.C. 113) [Total Sheets 3]
4. Oath or Declaration [Total Pages 3]
 - a. Newly executed (original or copy)
 - b. Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 16 completed)
 - i. DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

*NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

5. Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. Computer Readable Copy
 - b. Paper Copy (identical to computer copy)
 - c. Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. Assignment Papers (cover sheet & document(s))
8. 37 C.F.R. § 3.73(b) Statement Power of (when there is an assignee) Attorney
9. English Translation Document (if applicable)
10. Information Disclosure Statement (IDS)/PTO-1449 Copies of IDS Citations (2)
11. Preliminary Amendment
12. Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
 - * Small Entity Statement(s) Statement filed in prior application, (PTO/SB-09-12) Status still proper and desired
13. Certified Copy of Priority Document(s)
(if foreign priority is claimed)
14. Other: Check for \$345.00

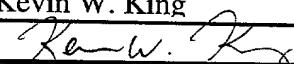
16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

Continuation Divisional Continuation-in-part (CIP) of prior application No: 08,548,840
Prior application information: Examiner Lilling, H. Group / Art Unit: 1651

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

<input checked="" type="checkbox"/> Customer Number or Bar Code Label	 (Insert Customer Number or Bar code label here) 23579		or	<input type="checkbox"/> Correspondence address below
Name	Patrea L. Pabst ARNALL GOLDEN & GREGORY, LLP 2800 ONE ATLANTIC CENTER 1201 WEST PEACHTREE STREET			
Address	Atlanta	State	GA	Zip Code
City	United States	Telephone	(404)873-8794	Fax (404) 873-8795

Name (Print/Type)	Kevin W. King	Registration No. (Attorney/Agent)	42,737
Signature		Date	06/12/00

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

MBX 012 CON 20061/18

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: David P. Martin, Oliver P. Peoples, and Simon F. Williams

Serial No.: Continuation of 08/548,840 Express Mail Label
No.: EL 381 208 545 US

Filed: June 12, 2000 Date of Deposit: June 12, 2000

**For: METHODS FOR ISOLATING POLYHYDROXYALKANOATES
 FROM PLANTS**

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

**REQUEST FOR FILING A CONTINUATION APPLICATION
UNDER 37 C.F.R. § 1.53(b)**

Sir:

This is a request for filing a continuation application under 37 C.F.R. § 1.53(b) of pending prior application Serial No. 08/548,840, filed October 26, 1995, by David P. Martin, Oliver P. Peoples, and Simon F. Williams, for "*Methods for Isolating Polyhydroxyalkanoates from Plants*". The application includes 1 page of Abstract, 22 pages of specification, 6 pages of claims, 3 sheets of formal drawings, and an executed Declaration of Inventorship from prior application U.S.S.N. 08/548,840.

A Preliminary Amendment, Information Disclosure Statement, 5 pages of Form
PTO-1449, and copies of two newly cited references are enclosed.

Also enclosed are copies from prior application U.S.S.N. 08/548,840 of (1) Assignment
from David Martin, Oliver Peoples, and Simon Williams to Metabolix, Inc., recorded
October 26, 1995, at Reel 7788, Frame 0897; (2) executed Verified Statement (Declaration)

Continuation of U.S.S.N. 08/548,840
"METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS"
Filed: June 12, 2000
Express Mail Label No. EL 381 208 545 US
Date of Deposit: June 12, 2000
REQUEST FOR FILING A CONTINUATION APPLICATION
UNDER 37 C.F.R. § 1.53(b)

Claiming Small Entity Status (37 C.F.R. §§ 1.9(f) and 1.27(c) --- Small Business Concern) by Metabolix, Inc.; and (3) executed Statement Under 37 C.F.R. § 3.73(b) by assignee Metabolix, Inc.

The inventorship for the claims in the continuation application is the same as in the prior application.

An Associate Power of Attorney under 37 C.F.R. § 1.34 is enclosed.

Submitted with the new application is a check in the amount of \$345.00 to cover the filing fee for a small entity. It is believed that this is the proper filing fee since the application includes 1 independent claim and a total of 8 claims, following entry of the enclosed Preliminary Amendment. This application is entitled to small entity status since a Verified Statement Claiming Small Entity Status was filed in prior Application Serial No. 08/548,840, a copy of which is submitted with this application, and small entity status is still proper (37 C.F.R. § 1.27(a)(1)).

This application is being filed on June 12, 2000 by mailing the application to Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231 via the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10.

The Express Mail label number appears in the heading of this paper, which is attached to the application papers pursuant to 37 C.F.R. § 1.10(b).

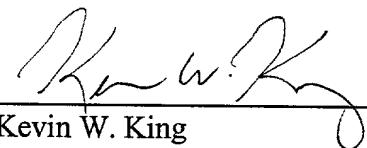
The Commissioner is hereby authorized to charge any fees that may be required, or credit any overpayment to Deposit Order Account No. 01-2507. To facilitate this process, applicants have enclosed a duplicate of this letter.

Continuation of U.S.S.N. 08/548,840
"METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS"
Filed: June 12, 2000
Express Mail Label No. EL 381 208 545 US
Date of Deposit: June 12, 2000
REQUEST FOR FILING A CONTINUATION APPLICATION
UNDER 37 C.F.R. § 1.53(b)

All correspondence concerning this application should be mailed to:

Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450

Respectfully submitted,



Kevin W. King
Reg. No. 42,737

Date: June 12, 2000

ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450
(404) 873-8596 (Telephone)
(404) 873-8597 (Fax)

Continuation of U.S.S.N. 08/548,840
"METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS"
Filed: June 12, 2000
Express Mail Label No. EL 381 208 545 US
Date of Deposit: June 12, 2000
REQUEST FOR FILING A CONTINUATION APPLICATION
UNDER 37 C.F.R. § 1.53(b)

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.10

I hereby certify that this Request for Filing a Continuation Application Under 37 C.F.R. § 1.53(b), and any documents referred to as attached therein are being deposited with the United States Postal Service on this date, June 12, 2000, in an envelope as "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10, mailing label number EL 381 208 545 US, addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231.



Teresa R. Spratt

Date: June 12, 2000

Applicant or Patentee: David P. Martin, et al. Attorney's
Serial or Patent No.: Docket No.:
MBX012

Filed or Issued: Herewith

For: METHODS FOR ISOLATING POLYHYDROXYALKANOATES
FROM PLANTS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am an official of the small business concern empowered to act on behalf of the concern identified below:

Metabolix, Inc.
303 Third Street, 2nd Floor
Cambridge, MA 02142-1196

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled "Methods for Isolating Polyhydroxyalkanoates from Plants" by David P. Martin, Oliver P. Peoples and Simon F. Williams described in the application filed herewith.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

"Methods for Isolating Polyhydroxyalkanoates from Plants"

By: David P. Martin, et al.

Filed: Herewith

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS

NAME **METABOLIX INC.**

ADDRESS **303 THIRD STREET, CAMBRIDGE, MA 02142**

INDIVIDUAL SMALL BUSINESS CONCERN NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING:

SIMON F. WILLIAMS

TITLE OF PERSON OTHER THAN OWNER

TREASURER

ADDRESS OF PERSON SIGNING

METABOLIX INC. 303 THIRD ST CAMBRIDGE

MA 02142

Simon F. Williams

SIGNATURE

0124195

DATE

CERTIFICATE UNDER 37 CFR 3.73(b)

Applicants: David P. Martin, et al.

Application No.:

Filed: Herewith

For: METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS

Metabolix, Inc.

(Name of Assignee)

a corporation

(Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

certifies that it is the assignee of the entire right, title and interest in the patent application identified above by virtue of either:

- A. An assignment from the inventor of the patent application identified above. The assignment was recorded in the Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

OR

- B. A chain of title from the inventor(s), of the patent application identified above, to the current assignee as shown below:

1. From:

To:

The document was recorded in the Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

2. From:

To:

The document was recorded in the Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

3. From:

To:

The document was recorded in the Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

Additional documents in the chain of title are listed on a supplement sheet.

Copies of assignments or other documents in the chain of title are attached.

The undersigned has reviewed all the documents in the chain of title of the patent application identified above and, to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned (whose title is supplied below) is empowered to act on behalf of the assignee.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : 10/24/95

Name : SIMON F. WILLIAMS

Title : TREASURER

Signature : SIMON F. WILLIAMS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: David P. Martin, Oliver P. Peoples, and Simon F. Williams

Serial No.: Continuation of 08/548,840

Express Mail Label No.:
EL 381 208 545 US

Filed: June 12, 2000

Date of Deposit:
June 12, 2000

For: METHOD FOR ISOLATING POLYHYDROXYALKANOATES
FROM PLANTS

BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examination, please amend the application as follows:

In the Specification

At page 1, line 3, please insert the following heading and paragraph:

--Cross-Reference To Related Applications

This is a continuation of U.S. Serial No. 08/548,840 filed October 26, 1995.--

In the Claims

Please cancel claims 1-35 and add the following new claims.

36. A method for isolating a polyhydroxyalkanoate from a biomass derived from a transgenic plant comprising the polyhydroxyalkanoate, the method comprising:

Continuation of U.S.S.N. 08/548,840

Filed: June 12, 2000

Express Mail Label No.: EL 381 208 545 US

Date of Deposit: June 12, 2000

PRELIMINARY AMENDMENT

- (a) treating the biomass, with at least one chemical or biochemical agent, to chemically derivatize the polyhydroxyalkanoate; and
- (b) separating the derivatized polyhydroxyalkanoate from the biomass.

37. The method of claim 36 wherein, in step (a), the biomass is treated with at least one chemical agent selected from the group consisting of acids, bases, detergents, oxidizing agents, chelating agents, reducing agents, nucleophilic reagents, electrophilic reagents, metal ions, aqueous solutions, and organic solutions.

38. The method of claim 36 wherein, in step (a), the polyhydroxyalkanoate is derivatized by a chemical transformation selected from the group consisting of an esterification, transesterification, hydrolysis, saponification, aminolysis, thiolysis, etherification, silylation, addition, elimination, rearrangement, and a condensation.

39. The method of claim 36 wherein the biochemical agent is an enzyme.

40. The method of claim 39 wherein the enzyme is selected from the group consisting of depolymerases, proteases, nucleases, lipases, cellulases, phosphorylases, and glycosidases.

41. The method of claim 36 wherein, in step (b), the derivatized polyhydroxyalkanoate is separated by a physical process selected from the group consisting of distillation, extraction, centrifugation, filtration, and chromatography.

42. The method of claim 36 wherein the transgenic plant is an oilseed plant.

43. The method of claim 36 wherein the polyhydroxyalkanoate includes one or more units selected from the group consisting of a 3-hydroxyacid, a 4-hydroxyacid, and a 5-hydroxyacid.

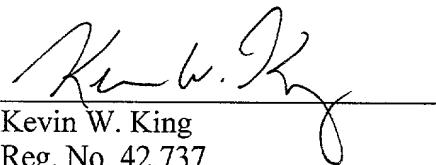
Continuation of U.S.S.N. 08/548,840
Filed: June 12, 2000
Express Mail Label No.: EL 381 208 545 US
Date of Deposit: June 12, 2000
PRELIMINARY AMENDMENT

Remarks

Claims 1-35 were cancelled, and new claims 36-43 were added. Support for the new claims is found in the specification, for example, at page 4, line 26 to page 5, line 4; page 9, lines 22-25; page 10, lines 2-6; page 17, line 6 to page 18, line 17; and in the claims as originally filed. No new matter has been added.

Allowance of claims 36-43 is earnestly solicited.

Respectfully submitted,



Kevin W. King
Reg. No. 42,737

Date: June 12, 2000

ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450
(404) 873-8596
(404) 873-8597 (Fax)

APPLICATION

FOR

UNITED STATES LETTERS PATENT

BY

DAVID MARTIN

OLIVER PEOPLES

AND

SIMON WILLIAMS

FOR

METHODS FOR ISOLATING

POLYHYDROXYALKANOATES FROM PLANTS

METHODS FOR ISOLATING
POLYHYDROXYALKANOATES FROM PLANTS

Background of the Invention

5

The present invention is generally in the area of isolating polyesters from plants.

Polyhydroxyalkanoates (PHAs) are a class of naturally occurring polyesters that are synthesized by numerous organisms in response to environmental stress. For reviews, see Byrom, D., "Miscellaneous Biomaterials", in D. Byrom, Ed., "Biomaterials" MacMillan Publishers, London, 1991, pp. 333-359; Hocking, P.J. and Marchessault, R.H., "Biopolymers," in G.J.L. Griffin, Ed., "Chemistry and Technology of Biodegradable Polymers", Chapman and Hall, London, 1994, pp. 48-96; Holmes, P.A., "Biologically Produced (R)-3-hydroxyalkanoate Polymers and Copolymers," in D.C. Bassett, Ed., "Developments in Crystalline Polymers," Elsevier, London, Vol. 2, 1988, pp. 1-65; Lafferty *et al.*, "Microbial Production of Poly- β -hydroxybutyric acid," H.J. Rehm and G. Reed Eds., "Biotechnology", Verlagsgesellschaft, Weinheim, Vol. 66, 1988, pp. 135-176; Müller and Seebach, *Angew. Chem. Int. Ed. Engl.*, 32:477-502 (1993); and Steinbüchel, A., "Polyhydroxyalkanoic Acids," Byrom, D., Ed., "Biomaterials", MacMillan Publishers, London, 1991, pp. 123-213.

The PHA biopolymers can be divided into two groups according to the length of their side chains (Figure 1). Those with short side chains (Figure 1a), such as polyhydroxybutyrate (PHB), a homopolymer of R-3-hydroxybutyric acid units, are crystalline thermoplastics, whereas PHAs with long side chains (Figure 1b) are more elastomeric. The former have been known for about seventy years (Lemoigne and Roukhelman, *Annales des Fermentations*, 5:527-536 (1925)) whereas the latter materials were first

identified in the early 1980's. De Smet *et al.*, *J. Bacteriol.*, 154:870-878 (1983).

Due to their earlier discovery and their desirable physical properties, the short side chain materials have been more extensively studied. The PHA polymers, which are natural thermoplastics, can be processed using conventional polymer technology and have industrially useful properties, such as biodegradability in soil and marine environments, biocompatibility, and good barrier properties. These characteristics make these materials useful for a wide range of industrial applications.

- The PHA polymers may constitute up to 90% of the dry cell weight of bacteria, and are found as discrete granules inside the bacterial cells. These PHA granules accumulate in response to nutrient limitation and serve as carbon and energy reserve materials. Distinct pathways are used by microorganisms to produce each class of these polymers. The pathway leading to the short side chain polyhydroxyalkanoates (SSCPHAs) involves three enzymes, thiolase, reductase and PHB synthase (sometimes called polymerase). Using this pathway, the homopolymer PHB is synthesized by condensation of two molecules of acetyl-Coenzyme A to give acetoacetyl-Coenzyme A, followed by reduction of this intermediate to R-3-hydroxybutyryl-Coenzyme A, and subsequent polymerization (Figure 2a). The last enzyme in this pathway, the synthase, has a substrate specificity that can accommodate C3-C5 monomeric units including R-4-hydroxy acid and R-5-hydroxy acid units. This biosynthetic pathway is found, for example, in the bacteria *Zoogloea ramigera* and *Alcaligenes eutrophus*.
- The biosynthetic pathway which is used to make the long side chain polyhydroxyalkanoates (LSCPHAs) is still partly unknown, however, it is currently thought that the monomeric hydroxyacyl units leading to the LSCPHAs are derived by the β -oxidation of fatty acids and the fatty acid

pathway (Figure 2b). The R-3-hydroxyacyl-coenzyme substrates resulting from these routes then are polymerized by PHA synthases that have substrate specificities favoring the larger monomeric units in the C6-C14 range. Long side chain PHAs are produced, for example, by *Pseudomonads*.

5 The biosynthesis of PHAs has been studied in a wide range of bacteria at both the biochemical and genetic level, and has been reviewed in Steinbuchel *et al.*, *FEMS Microbiology Reviews*, 103:217-230 (1992). Since the first PHA synthase genes were identified and characterized in 1989 (Peoples and Sinskey, *J. Biol Chem.*, 264:15298-15303 (1989); and U.S. Patent Nos. 5,229,279, 5,245,023, and 5,250,430 to Peoples and Sinskey), a number of other microbial PHA polymerases have been investigated and their DNA and amino acid sequences published. Steinbuchel *et al.*, *FEMS Microbiology Reviews*, 103:217-230 (1992). More recently, two subunit PHA synthases from *Chromatium vinosum* (Liebersgesell, M. and

10 15 Steinbuchel, A., *European J. Biochem.*, 209:135-150 (1992); and WO 93/02194) and *Thiocystis violacea* (Liebersgesell, M. and Steinbuchel, A., *Appl. Microbiol. Biotechnol.* 38:493-501 (1993)) have been described.

20 The genes encoding the enzymes responsible for the production of SSCPHAs in, for example, *Z. ramigera* and *A. eutrophus*, have been isolated and sequenced. Peoples and Sinskey, *Prog. Biotechnol.* 3:51-56 (1987); Peoples *et al.*, *J. Biol. Chem.*, 262:97-102 (1987); Peoples and Sinskey (1989), *J. Biol. Chem.* 264:15298-15303, *J. Biol. Chem.* 264:15293-15297, and *Molecular Microbiol.* 3:349-357; Slater *et al.*, *J. Bacteriol.*, 170:4431-4436 (1988); and Schubert *et al.*, *J. Bacteriol.*, 170:5837-5847 (1988).

25 PHA producing microorganisms produce PHA to greater than 60% total dry weight and are readily extractable by organic solvent. Lafferty *et al.*, "Microbial Production of Poly- β -Hydroxybutyric Acid", in H.J. Rehm and G. Reed, Eds., "Biotechnology", Verlagsgesellschaft, Weinheim, Vol. 66, 1988, pp. 135-176. In plants, the extraction and recovery of PHA is

significantly complicated by the presence of large amounts of plant oil as well as lower percentages of PHA. These complicating factors make the successful extraction, separation and recovery of PHAs from plants more difficult.

5 There is a need for the development of methods for the large scale processing and purification of polyhydroxyalkanoates from plant biomass. It is therefore an object of the invention to provide methods for processing PHAs from plant biomass on a large scale. It is another object of the invention to provide methods for isolating PHAs from transgenic oil crop plants. It is a further object of the invention to provide methods for processing plant biomass derived from oil seed crop plants such that the recovery of the non-PHA products such as plant oils also is maximized.

10

Summary of the Invention

15

Methods are provided for separating a polyhydroxyalkanoate ("PHA") from plants. In one embodiment, methods are provided for isolating PHAs from a plant biomass derived from transgenic crop plants which contain plant oils. The methods advantageously permit both the oil and the PHAs to be recovered from the plant biomass. To isolate a PHA, in one embodiment, a biomass derived from an oil crop plant is pre-processed, for example by grinding, crushing or rolling. The oil then is extracted from the biomass with a first solvent in which the oil is soluble and in which the PHA is not highly soluble, to separate the oil from the PHA. The essentially oil-free plant biomass then is extracted with a second solvent in which the PHA is soluble, to separate the PHA from the biomass. Alternatively, the PHA-containing biomass is treated with a chemical or biochemical agent, such as an enzyme, to chemically transform the PHA into a PHA derivative. The derivatized PHA then is separated from the mixture using, for example, a

20

25

RECEIVED
U.S. PATENT AND TRADEMARK OFFICE

physical separation process such as distillation, extraction or chromatography. Advantageously, using the method, plant oils, PHAs, and PHA derivatives all can be recovered and purified on a large scale from plants such as transgenic oil crop plants.

5

Brief Description of the Drawings

Figure 1a is an illustration of the structure of short side chain polyhydroxyalkanoates.

10 Figure 1b is an illustration of the structure of long side chain polyhydroxyalkanoates.

Figure 2a is an illustration of a biosynthetic pathway for the synthesis of the short side chain polyhydroxyalkanoate, polyhydroxybutyrate.

15 Figure 2b is an illustration of a biosynthetic pathway for the synthesis of long side chain polyhydroxyalkanoates.

Figure 3 is flow chart illustrating one embodiment of a process for separating polyhydroxyalkanoates from plants.

Figure 4 is a flow chart illustrating another embodiment of a process for separating polyhydroxyalkanoates from plants.

20

Detailed Description of the Invention

Methods are provided for separating polyhydroxyalkanoates ("PHAs") from a plant biomass containing plant oil and meal. PHAs which can be isolated from plant biomass include degradation or other products of PHAs, such as monomers, dimers, oligomers, acids, esters, amides, and lactones, which can be formed from chemical, biochemical or physical treatment during processing of the biomass, or from processes occurring within the plant biomass. In a preferred embodiment, the PHAs are isolated from a

25

biomass derived from a transgenic oil crop plant. In addition to maximizing the recovery of PHA materials, the recovery of commercially useful non-PHA products from the biomass also is maximized. For example, in the case of PHA separation from the seed of an oil-seed plant, the oil and meal also can be isolated and then used commercially.

5

I. Materials for Isolation of PHAs

A. PHA Materials which can be Isolated

PHA materials which can be isolated from plant biomass include monomers, polymers and other products derived from PHAs including chemically and biologically modified derivatives. The PHA materials are defined in one embodiment as containing one or more units, for example 10 to 100,000 and preferably 100-30,000 units of the following formula I:

10



15

wherein n is 0 or an integer, for example 1-15, and in a preferred embodiment 1-4; and

20

wherein R¹, R², R³, and R⁴ independently can be hydrocarbon radicals including long chain hydrocarbon radicals; halo- and hydroxy-substituted radicals; hydroxy radicals; halogen radicals; nitrogen-substituted radicals; oxygen-substituted radicals; and/or hydrogen atoms.

As defined herein, the formula -(CR³R⁴)_n- is defined as including but not limited to the following formulas:

25

-CR³R⁴- (where n=1);

-CR³R⁴CR^{3'}R^{4'}- (where n=2); and

-CR³R⁴CR^{3'}R^{4'}CR^{3''}R^{4''-} (where n=3);

wherein R³, R⁴, R^{3'}, R^{4'}, R^{3''}, and R^{4''} can be independently hydrocarbon radicals including long chain hydrocarbon radicals; halo- and

hydroxy-substituted radicals; hydroxy radicals; halogen radicals; nitrogen-substituted radicals; oxygen-substituted radicals; and/or hydrogen atoms. Thus, formula I includes units derived from 3-hydroxyacids (n=1), 4-hydroxyacids (n=2), 5-hydroxyacids (n=3).

These units may be the same, as in a homopolymer, or be selected from two or more different units, as in a copolymer or terpolymer. The polymers in one embodiment have a molecular weight above 300, for example 300-10⁷, and in a preferred embodiment, 10,000 to 3,000,000 Daltons. In one embodiment, PHA homopolymers may be isolated such as, for example, polyhydroxybutyrate, polyhydroxyvalerate or polylactic acid. Additionally, PHA copolymers or terpolymers including at least two monomers of a hydroxyalkanoate such as hydroxybutyrate, hydroxyvalerate, hydroxyhexanoate, hydroxyheptanoate, hydroxyoctanoate, hydroxynonanoate and hydroxydecanoate can be isolated. PHAs including monomers and polymers and derivatives of 3-hydroxyacids, 4-hydroxyacids and 5-hydroxyacids can be isolated.

The PHA polymers also may contain or be modified to include other non-hydroxy acid units such as long chain fatty acids, amino acids, carbohydrates, phosphorus and sulfur containing compounds, and triols, such as glycerol. PHA products which can be isolated include derivatives formed upon physical, chemical or biochemical treatment of the biomass or by processes within the biomass including hydroxyacid monomers, dimers, trimers, linear and cyclic oligomers and lactones. PHA derivative products which can be isolated include esters, diols, unsaturated compounds, aldehydes, acids, alcohols, lactones, cyclic and linear esters, amides, and thioesters of polyhydroxyalkanoates or of a monomer derived from the polyhydroxyalkanoate.

Representative PHA products which can be isolated from plant biomass include:

esters defined by the formula: HO $R^1R^2(CR^3R^4)_nCO_2R^5$;

amides defined by the formula: HO $R^1R^2(CR^3R^4)_nCONR^5R^6$;

5 thioesters defined by the formula: HO $R^1R^2(CR^3R^4)_nCOSR^5$;

acids defined by the formula: HO $R^1R^2(CR^3R^4)_nCO_2H$;

ethers defined by the formula: R $^6OCR^1R^2(CR^3R^4)_nCO_2R^5$;

esters defined by the formula: R $^6CO_2CR^1R^2(CR^3R^4)_nCO_2R^5$;

unsaturated compounds defined by the formulas:

10 R $^1R^2C=CR^3(CR^4R^5)_nCO_2R^6$; and R $^1R^2R^3CC(R^4)=C(R^5)CO_2R^6$;

diols defined by the formula: HO $R^1R^2(CR^3R^4)_nCH_2OH$;

lactones or macrolides, defined by the formula:

{ -OCR $^1R^2(CR^3R^4)_nCO-$ }_x ;

15 where x is an integer, for example from 1 to 10; and

ketones or aldehydes defined by the formula:

HO $R^1R^2(CR^3R^4)_nCOR^5$;

HO $R^1R^2(CR^3R^4)_nCHO$;

R $^1CO(CR^2R^3)_nCOOR^4$; and

20 R $^1CO(CR^2R^3)_nCOR^4$;

wherein n is 0 or an integer; and

wherein R 1 , R 2 , R 3 , R 4 , R 5 and R 6 are each independently

hydrocarbon radicals including long chain hydrocarbon radicals, halo- and

hydroxy-substituted radicals, hydroxy radicals, halogen radicals, nitrogen-

25 substituted radicals, oxygen-substituted radicals, and hydrogen atoms; and

wherein -(CR $^2R^3)$ _n- is defined as described above.

Commercially useful monomer PHA products such as 3-hydroxybutyric acid or crotonic acid, or alkyl esters thereof, including methyl-3-hydroxybutanoate, ethyl-3-hydroxybutanoate, propyl-3-

hydroxybutanoate and butyl-3-hydroxybutanoate also can be isolated. The PHA derived hydroxy acid monomers, in addition to the higher molecular weight forms, are a source of valuable chemicals that can be used commercially either with or without further modification.

5 As used herein, the term "PHA materials", or "PHAs" or "polyhydroxyalkanoates" refers to monomers, polymers and other PHA-based materials originally present in the biomass prior to processing, and products formed during processing such as products formed from degradation or processes occurring within the plant biomass or derivative products formed
10 by treatment of the biomass with chemical or biological agents to cause a chemical transformation.

B. Plant Sources From Which PHAs Can Be Isolated

Plant Species

PHAs and PHA products can be isolated from plant biomass derived
15 from plants such as soybean, cotton, coconuts, groundnuts, rapeseed, sunflower seed, olive, palm, sesame seed, linseed, castor, safflower seed, tobacco and potato. In a preferred embodiment, the biomass can be derived from an oil crop plant, particularly rapeseed, sunflower seed, safflower seed, linseed and soybean. In the processing, the plant oil in the seed crop plants
20 can be isolated and recovered in addition to the PHA polymers. Plant oils typically make up 10-50% of the seed by weight. The worldwide demand for plant oil is considerable. The methods for processing the plant biomass can be tailored based on the properties of the particular PHA polymer or derivative being isolated, and based on the type of plant crop and the plant
25 components being extracted.

Production of Transgenic Plants

The use of transgenic oil crop plants offers many advantages. Transgenic crop plants for production of PHAs can be obtained using methods available in the art. Transgenic plant crop production can produce

PHA polymers at both a price and a scale that is competitive with petrochemical derived plastics. Transgenic plant derived PHA polymers or their derivatives can be processed and separated from plant biomass in commercially useful forms. The location of the PHA in the plant crop (*e.g.*, leaf, seed, stem or combinations thereof) can be varied to maximize the yield of PHA from the plant.

The genes encoding the enzymes responsible for the production of short side chain PHAs in, for example, *Z. ramigera* and *A. eutrophus*, have been identified, isolated and sequenced. Peoples and Sinskey, *Prog. Biotechnol.* 3:51-56 (1987); Peoples *et al.*, *J. Biol. Chem.*, 262:97-102 (1987); Peoples and Sinskey (1989), *J. Biol. Chem.* 264:15298-15303, *J. Biol. Chem.* 264:15293-15297, and *Molecular Microbiol.* 3:349-357; Slater *et al.*, *J. Bacteriol.*, 170:4431-4436 (1988); and Schubert *et al.*, *J. Bacteriol.*, 170:5837-5847 (1988), the disclosure of which is incorporated herein by reference. In *A. eutrophus*, they were found to form an operon, *phbC-phbA-phbB* genes, coding for the short side chain PHA synthase, thiolase, and reductase, respectively. For the long side chain PHAs, the synthase enzymes in the *Pseudomonas* organism were found to be encoded by the *pha* locus. This locus includes two closely related PHA synthase genes, *phaA* and *phaC*, as well as a depolymerase gene which is the product of the *phaB* gene.

Methods which can be used for producing PHA polymers in transgenic crop species are described in: U.S. Patent Nos. 5,245,023 and 5,250,430; WO 91/00917; WO 92/19747; WO 93/02187; WO 93/02194; WO 94/11519; WO 94/12014; WO 94/23027; WO 95/05472; Poirier *et al.*, *Science*, 256:520-523 (1992), Poirier *et al.*, *Bio/Technol.*, 13:142-150 (1995) and Nawrath *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:12760-12764 (1994), the disclosures of which are incorporated herein by reference.

To form a transgenic crop species, a gene encoding a PHA synthase is transferred from a microorganism into plant cells to obtain the appropriate level of production of the PHA synthase enzyme. The gene may be derived from a microorganism such as *Acinetobacter*, *Aeromonas*, *Alcaligenes*,
5 *Azotobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Chromatium*,
Flavobacterium, *Halobacterium*, *Pseudomonads*, *Nocardia*, *Rhodococcus*,
Thiocystis, *Streptomyces*, *Streptococcus* or *Zoogloea*. Additional PHA
biosynthetic genes also can be provided, for example, an acetoacetyl-CoA
reductase gene or other genes encoding enzymes required to synthesize the
10 substrates for the PHA synthase enzymes. The expression in different plant
tissues or organelles can be controlled using methods known to those skilled
in the art. Gasser and Fraley, *Science*, 244:1293-1299 (1989), the disclosure
of which is incorporated herein by reference, and references cited therein.
PHB has been produced in genetically engineered plant systems by standard
15 techniques. Poirier, Y. *et al.*, *Science*, 256:520-523 (1992); Poirier, Y. *et*
al., *Bio/Technol.*, 13:142-150 (1995); and Nawrath, C. *et al.*, *Proc. Natl.*
Acad. Sci. USA, 91:12760-12764 (1994), the disclosures of which are
incorporated herein by reference.

In a preferred embodiment, the PHA content of the plant biomass
20 prior to extraction is at least 1% by weight of the dry weight of biomass,
more preferably 5-95% by weight of the dry weight of biomass, and in
another preferred embodiment between about 5-60% by weight of the dry
weight of biomass, most preferably 10-60%. Preferably, at least 24% of the
PHA is recovered in the process separate from oil.

II. Methods For Isolation Of PHAs From Plants

A. Pre-processing of the Plant Biomass

The PHA-containing plant biomass, for example, a transgenic oil crop plant containing a heterologous PHA synthase gene, is cultivated and harvested. The plant biomass may be pre-processed prior to extraction of the PHA polymers using methods available in the art, such as agitation, heating, cooling, pressure, vacuum, sonication, centrifugation, and/or radiation. As used herein, the term "plant biomass" refers to plant components including seeds, leaf, stalk and stem. Additionally, the plant biomass can be pre-processed using any one or more combinations of procedures including drying, dehulling, cleaning, ageing, cleaning, weighing, cracking, flaking, pressing, rolling, grinding, cooking, crushing, settling, and/or filtering. The use of these procedures for separating oil from meal in the processing of oil bearing plants is described in "Oil Crops of the World," G. Röbbelen et al., Eds., McGraw-Hill Publishing Company, 1989, Chapter 11, the disclosure of which is incorporated herein by reference.

B. Extraction of Plant Biomass

The PHA monomers, polymers and derivatives can be removed from the plant biomass using suitable means including solvent extraction and/or washing, aqueous extraction and/or washing, crushing, temperature treatment, enzymatic treatment, centrifugation, supercritical fluid extraction, high and/or reduced pressure treatment, chromatography, distillation, melting, or treatment with reagents to facilitate separation of the PHA materials.

Methods for extracting the oil from the pre-processed material available in the art also may be used, such as oil expeller pressing where the oil is mechanically squeezed from the oil bearing material, and prepressing solvent extraction where a portion of the oil is removed by expellers and the remainder by extraction with an organic solvent, such as a hydrocarbon, for

example, hexane. Additionally, supercritical gases including carbon dioxide and propane can be used. Other methods include direct solvent extraction where the oil is removed directly from conditioned seed with an organic solvent; propane refining to separate fat; and fat splitting involving hydrolysis of fat or oil with water to produce glycerol and fatty acid. "Oil Crops of the World," G. Röbbelen *et al.*, Eds., McGraw-Hill Publishing Company, 1989; "Liquid Extraction," R. Treybal, Ed., McGraw-Hill Book, New York, 1951; and "World Oilseeds: Chemistry, Technology, and Utilization," D.K. Salunkhe *et al.*, Eds., Van Nostrand Reinhold, New York, 1992.

- 10 Extraction of Oil from Plant Biomass
- One preferred method for isolating the PHAs from a plant biomass is illustrated in the flow chart of Figure 3. In the process, the PHA containing plant biomass first optionally is pre-processed as described above. The pre-processed or unprocessed PHA containing plant biomass then is extracted in a solvent in which the oil is soluble, and in which the PHA and the meal are not highly soluble, to remove the majority or all of the oil from the PHA containing plant biomass. The solvent is selected such that it is a good extractant for the oil and a poor extractant with low solubility for the PHA and the plant meal. Extraction of the PHA-oil-meal mixture, as illustrated in the flow chart of Figure 3, produces an oil fraction essentially free of PHA (for example including less than about 10% by weight of PHA) and an essentially oil free PHA-meal mixture (including for example, less than about 10% oil by weight). The PHA-meal mixture then is extracted with a second solvent in which the PHAs are soluble, to obtain purified PHA materials.
- 15
- 20
- 25 Alternatively, the PHA-meal mixture can be treated chemically or enzymatically to produce PHA derivatives which are then isolated from the meal, as illustrated in Figure 3.

The first solvent which is used to extract the oil from the plant biomass is selected based on its ability to solubilize the oil. Preferably, a

solvent is used in which the oil is soluble and in which the PHA and plant material is not highly soluble. Suitable solvents include hydrocarbons, such as propane, butane, pentane, hexane, heptane, octane, nonane and decane.

As used herein the term "solvent" includes solvents as well as solvent mixtures, such as mixtures of hydrocarbons. Preferably, the first solvent is chosen wherein the PHA is soluble to less than 1%, most preferably less than 0.1% and the oil is soluble to more than 10% (w/v, ambient temperature).

To isolate the PHA and oil components from the biomass, solvents used in the extractions are selected which exploit the differences in the physical nature and solubility characteristics of the PHA and oil components of the biomass. The isolation steps are tailored depending on the particular PHA, plant host or PHA/plant host combination. For example, in the extraction of PHB and LSCPHA, different solvents or solvent combinations are used in their extraction from PHA-containing transgenic plant biomass based on their solubility.

In the embodiment where the PHA is separated from the PHA-meal product by treatment with a second solvent, the second solvent (solvent 2 in Figure 3) is selected based on its capability of being a good extractant for the PHA and a poor extractant for the meal. Solvents which can be used include solvents or solvent mixtures including chloronated organic solvents such as chloroform, methylene chloride, dichloroethane, trichloroethane, tetrachloroethane and dichloroacetate. For example, hydrocarbon stabilized chloroform can be used. Other solvents which have been used to extract PHAs from microbial sources which may be used include alkyl carbonates, such as propylene carbonate and ethylene carbonate, trifluoroethanol, acetic anhydride, dimethylformamide, ethylacetoacetate, triolein, toluene, dioxane, tetrahydrofuran, diethylether, pyridine, hydroxyacids and alcohols having more than 3 carbon atoms, as well as mixtures thereof. Lafferty *et al.*, "Microbial Production of Poly- β -Hydroxybutyric Acid," in H.J. Rehm and

G. Reed, Eds., "Biotechnology", Verlagsgesellschaft, Weinheim, Vol. 66, 1988, pp. 135-176. In a preferred embodiment, the second solvent is a chlorinated organic solvent or an alkyl carbonate. Additionally, in a preferred embodiment, the first and second solvents have boiling points between ambient temperature and 400°C, more preferably between 30°C and 250°C.

The solvent extraction steps also can be conducted using supercritical fluid extraction, wherein a gas is used such as ethylene, propylene, propylene oxide, butane or carbon dioxide. In a preferred embodiment, the gas has a boiling point between -250°C and ambient temperature, preferably between -150°C and -20°C. The PHA also may be extracted in a molten state.

In an alternative embodiment, as illustrated in the flow chart of Figure 3, the PHA-meal mixture is treated with a chemical or biochemical agent, such as an enzyme, to chemically transform the PHAs into PHA derivatives as described in detail below. The PHA derivatives then are separated from the plant biomass if necessary, using one or more subsequent physical separation steps such as distillation, extraction, centrifugation, filtration or chromatography.

Extraction of PHA and Oil.

In another embodiment, shown in the flow chart of Figure 4, the PHA containing plant biomass optionally first is pre-processed as described above. The pre-processed or unprocessed PHA containing plant biomass then is solvent extracted in a solvent in which the oil and the PHAs are soluble, and in which the meal is not highly or is poorly soluble, to essentially remove the oil and the PHAs from the plant meal, such that, for example, less than about 10% of oil and PHAs by weight remain in the plant meal. The solvent used in this process is selected such that it is a good extractant for the PHAs and oil, and a poor extractant for the meal. The PHA materials in the PHA-oil product then are further separated from the oil

by a physical separation step, such as distillation, or by further exploitation of differences in solubility between the PHA and oil.

Alternatively, the PHA-oil product may be modified by chemical or biological treatment to provide a PHA derivative(s)-oil product as described below (as shown in Figure 4). The PHA derivative component of the latter may be subsequently purified by physical processing, including distillation, solvent extraction, washing, precipitation, centrifugation, supercritical fluid extraction, filtration, and chromatography.

Solvents which may be used to extract the oil-PHA component from the plant biomass include chlorinated organic solvents, for example, chloroform, methylene chloride, di-, tri-, tetra-chloroethane and dichloroacetate, alkyl carbonates such as propylene carbonate and ethylene carbonate, trifluoroethanol, acetic anhydride, dimethylformamide, ethylacetoacetate, triolein, acetic acid, toluene, alcohols, hydroxyacids, dioxan, tetrahydrofuran, diethylether, and/or pyridine. The solvent also may consist of or may include hydrocarbons such as hexane, heptane, octane, nonane or decane or mixtures thereof. Preferred solvents are those having boiling points between ambient temperature and 400°C, preferably between 30°C and 250°C. Preferably, such solvents have solubility for both PHA and oil components of at least 5% (w/v, ambient temperature), and are chosen depending upon the structure of the PHA defined in Figure 1. The PHA material also can be extracted in the molten state. The choice of solvent will depend on the choice of plant from which the biomass is derived and the solubility properties of the PHAs, derivatives and oils being separated.

As illustrated in the flow chart of Figure 4, the extracted PHA-oil also can be separated by chemical modification to form a PHA derivative-oil product, by treatment with a chemical or biological agent, such as an enzyme which degrades the PHA material, as described in detail below. The PHA

derivative then is separated from the PHA derivative-oil mixture using, for example, a physical process such as distillation, extraction, centrifuation, supercritical fluid extraction, preparation filtration, and/or chromatography. Further refining of the essentially oil free PHA can be carried out by standard procedures known to those skilled in the art.

5 **III. Synthesis of PHA Derivatives**

As described above, during the processing, the PHA materials in the biomass can be derivatized by physical, chemical or enzymatic conversion into derivatives, prior to their isolation, to faciliate the isolation of the 10 materials, or to produce a desired derivative product. PHA derivatives which can be formed include acids, esters, oligomers, cyclic oligomers, lactones, macrolides, amides, amines, thioesters, diols, and unsaturated compounds, which can be formed using methods available in the art.

Griesbeck, A. and Seebach, D., *Helv. Chim. Acta* 70:1320-1325 (1987);
15 Plattner, D.A., *Helv. Chim. Acta*, 76:2004-2033 (1993); Seebach, D. *et al.*, "Biological-Chemical Preparation of 3-Hydroxycarboxylic Acids and Their Use in EPC-synthesis," W. Bartmann and K.B. Sharpless, Eds., "Stereochemistry of Organic and Bioorganic Transformations," VCH, Weinheim, 1987, pp. 85-126; and Seebach, D. *et al.*, *Chimia*, 44:112-116
20 (1990); *Org. Synth.*, 71:39-47 (1992); *Angew. Chem. Int. Ed. Eng.*, 434-435 (1992); and *Helv. Chim. Acta*, 77:1099-1123 (1994), the disclosures of which are incorporated herein by reference. Additional methods for derivatizing esters, which may be used to form PHA polyesters are known to those skilled in the art.

25 Chemical agents which can be used to modify the PHA materials in the processing of the biomass include, for example, acids, bases, detergents, chelator, an oxidizing or reducing agent, a nucleophilic or electrophilic reagent, metal ions, aqueous solutions or organic solutions, and free radicals. Other chemical agents which can be used include hydrogen peroxide,

hypochlorite, ozone and alkyl peroxides. The chemical transformation can be, for example, a chemical reaction such as an esterification, transesterification, hydrolysis, saponification, aminolysis, thiolysis, etherification, silylation, addition, elimination, rearrangement, or condensation. The chemical agents can be used, for example, to produce derivatives with a molecular weight less than that of the PHA starting materials in the plant biomass. Additionally, the PHA materials can be modified by physical treatment such as heat, cold or agitation.

The PHA materials also can be chemically modified during processing by treatment of biomass materials such as PHA-meal or PHA-oil mixtures with a biological agent such as an enzyme, which for example, degrades the biomass or the PHA material. Enzymes which can be used include PHA depolymerases, proteases, nucleases, lipases, hydratases, phosphorylases, cellulases and/or glycosidases. The PHA polymers may be converted to oligomers, monomers, dimers, trimers, or other derivatives. The PHA functionality may also be converted to non-PHA chemical functionality.

IV. Applications

The PHAs isolated as described herein can be used in a wide variety of different applications. In one embodiment, the isolated PHAs can be used to form a latex. PCT WO 91/13207 discloses the use of polymers or copolymers of β -hydroxybutyrate and β -hydroxyvalerate in the form of a latex, *i.e.*, as an aqueous suspension of non-crystalline, amorphous particles. The latex can be used, for example, to form films or coated papers which are biodegradable and recyclable. Methods for forming a PHA latex from purified crystalline PHAs are described in PCT WO 94/07940, the disclosure of which is incorporated herein by reference. In the method, a purified solution of PHA in an organic solvent, which can be obtained as described herein, is emulsified in an aqueous solution including a surfactant, to form an

amorphous latex. Thus, the methods disclosed herein provided purified PHAs which can be used in a variety of industrial and biomedical applications such as the formation of PHA latex materials.

The invention will be further understood from the following non-limiting examples.

**Example 1: Extraction of Polyhydroxybutyrate
from Plant Biomass.**

The process illustrated in Figure 3 was used to isolate polyhydroxybutyrate (PHB) from plant biomass by extraction with hexane (solvent 1) to remove the oil followed by extraction with hydrocarbon stabilized chloroform (solvent 2) to isolate PHB.

A sample of rapeseed (32 g) containing approximately 40 weight % oil was admixed with 6 g of PHB powder (Aldrich) and ground using an electric food grinder. This sample is representative of a transgenic oil seed containing 34% by weight oil and 16% by weight of PHB. The mixture was continuously extracted with 300 mL hexane (solvent 1) in a soxhlet apparatus for 6 hours after which time the sample was allowed to cool providing an organic solvent phase and a solid meal. The organic solvent was concentrated to yield a yellow oil (11.8 g, 31% by weight of the admixture). NMR analysis indicated that the oil contained no PHB. This result indicates that PHB-free oil can be readily recovered at greater than 90% yield from PHA containing plant biomass. A portion of the solid meal (7.7 g) was then further extracted with 120 mL of hydrocarbon stabilized chloroform (Solvent 2) for 22 hours in a soxhlet apparatus. Evaporation of the chloroform solution resulted in the formation of a yellow/white plastic film weighing 1.15 g. A portion of the crude PHB film (227 mg) was washed with three, one mL portions of hexane. After air drying, the PHB film (86 mg) was off-white in color. NMR analysis of this film indicated that it was essentially

pure PHB. The recovery of PHB film represents a 24% yield based on the original PHB content of the admixture.

Example 2: Extraction of a PHB Derivative from Plant Biomass.

5 The process illustrated in Figure 3 was used to isolate polyhydroxybutyrate (PHB) from plant biomass by extraction of plant biomass with hexane (solvent 1) to remove the oil fraction followed by chemical treatment and then physical separation of the PHB in derivative form.

10 A portion of the PHB containing residual meal (2.32 g) from Example 1 was heated at reflux for 15.5 hours with n-butanol (25 mL) and concentrated sulfuric acid (0.33 mL). To the resultant black mixture was added saturated sodium bicarbonate (20 mL), brine (20 mL) and ethyl acetate (50 mL). The mixture was shaken in a separatory funnel and the phases

15 were separated. The organic phase was filtered through a pad of celite, washed with brine, treated with a small amount of activated charcoal, filtered, and concentrated to a dark oil. This material was distilled under reduced pressure. The fraction distilling at 49-53°C and 0.25 torr was collected to yield a slightly yellow colored liquid (0.47 g). NMR analysis of

20 this material confirmed that it was butyl 3-hydroxybutyrate. The amount of material recovered represents a 46% yield of derivatized PHB based on the amount of PHB contained in the residual meal.

Example 3: Extraction of PHAs from Rapeseed

PHA was extracted in polymer form from rapeseed using the process 25 of Figure 4 as follows. A sample of rapeseed (20 g) containing approximately 40% by weight oil was admixed with small pieces of PHO, a copolymer including approximately 94% 3-hydroxyoctanoic acid and approximately 6% 3-hydroxyhexanoic acid (5.43 g, isolated from *Pseudomonas putida*) and ground using an electric food grinder. This sample

is representative of a transgenic oil seed containing 31% by weight oil and 21% by weight of PHO. The mixture was continuously extracted with 300 mL hexane (Solvent 1) in a soxhlet apparatus for 12 hours. The sample was allowed to cool and was filtered to provide an organic solvent phase and a solid meal (11.02 g after air drying). The solvent phase was concentrated to yield a yellow, very viscous gel-like material (12.92 g, 51% by weight of the admixture). Upon standing, this material set into a yellow, plastic-like solid. NMR analysis indicated that the material contained PHO and rapeseed oil. These results indicate that PHO oil mixture can be readily recovered at greater than 90% yield from PHA containing plant biomass. The PHA/oil mixture obtained was suitable for further purification.

Further purification was conducted as follows. A portion of the yellow, plastic-like material (0.136 g, approximately 41% wt PHO, 1.1 x 0.2 cm) was washed with 2 ml of n-propanol. After slowly swirling at room temperature for 3 days, the supernatant was removed, and the residual solid was washed overnight with 2 ml of methanol. The methanol wash was combined with the propanol wash and concentrated to yield a yellow oil (0.0876 g). After drying under vacuum, the residual solid polymer (0.048 g) was semi-transparent and almost colorless. This represents an 84% yield of PHO from the original rapeseed/PHO mixture. NMR analysis of the purified polymer showed that it was PHO (approximately 95% purity) containing a small amount of rapeseed oil. G.C. analysis showed a 10 fold reduction of major contaminants relative to the yellow, plastic-like material initially isolated by hexane extraction.

25 Example 4: Isolation of a PHA Derivative from Plant Biomass

The yellow PHO containing plastic-like material obtained prior to further purification, as described in Example 3, was further purified in derivative form by chemical treatment and physical separation: A portion of the partially purified PHO containing plastic material (2.75 g, containing

approximately 40% by weight PHO) isolated by hexane extraction from Example 3 was dissolved in n-butanol (50 mL) with heating. Concentrated sulfuric acid (0.7 mL) was added and the mixture was heated at reflux for 20 hours. After cooling to room temperature, saturated sodium carbonate (4 mL) was added to make the mixture basic to pH paper. The reaction mixture was filtered, the phases were separated and the organic layer washed with brine (2 x 30 mL). The organic phase was concentrated to about 10 mL, dissolved in chloroform (50 mL), dried over magnesium sulfate, filtered and concentrated under vacuum to a yellow oil (2.75 g). This material was distilled under reduced pressure. The fraction distilling at 93-97°C and 0.45 torr was collected to yield a clear, colorless liquid (0.41 g). The amount of material recovered represents a 25% yield of derivatized PHO based on the amount of PHO contained in the plastic-like starting material. NMR analysis of this material indicated that it is butyl 3-hydroxyoctanoate of approximately 95% purity and that it contains a very small amount of unsaturated material.

Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.

We claim:

1. A method for separating a polyhydroxyalkanoate ("PHA") from a plant biomass comprising plant oil and meal, the method comprising extracting the plant biomass with a first solvent to essentially remove the oil from the biomass, and then separating the PHA from the plant biomass.

2. The method of claim 1 wherein the plant biomass is derived from a transgenic plant.

3. The method of claim 1 further comprising derivatizing the PHA prior to separating the PHA from the biomass.

4. The method of claim 1 wherein the separated PHA comprises one or more units having the formula:



wherein n is 0 or an integer; and

wherein R¹, R², R³, and R⁴ each are independently selected from the group consisting of hydrocarbon radicals, halo- and hydroxy-substituted radicals, hydroxy radicals, halogen radicals, nitrogen-substituted radicals, oxygen-substituted radicals and hydrogen atoms.

5. The method of claim 4 wherein the separated PHA is selected from the group consisting of monomers, dimers, linear and cyclic oligomers, and lactones of the units.

6. The method of claim 3 wherein the separated PHA is selected from the group consisting of

esters defined by the formula: HOCR¹R²(CR³R⁴)_nCO₂R⁵;

amides defined by the formula: HOCR¹R²(CR³R⁴)_nCONR⁵R⁶;

thioesters defined by the formula: HOCR¹R²(CR³R⁴)_nCOSR⁵;

acids defined by the formula: HOCR¹R²(CR³R⁴)_nCO₂H;

ethers defined by the formula: R⁶OCR¹R²(CR³R⁴)_nCO₂R⁵;

esters defined by the formula: R⁶CO₂CR¹R²(CR³R⁴)_nCO₂R⁵;

unsaturated compounds, defined by the formulas:

$R^1R^2C=CR^3(CR^4R^5)_nCO_2R^6$; and

$R^1R^2R^3CC(R^4)=C(R^5)CO_2R^6$;

diols defined by the formula: $HOCR^1R^2(CR^3R^4)_nCH_2OH$;

lactones or macrolides defined by the formula:

$\overbrace{-OCR^1R^2(CR^3R^4)_nCO-}^{\text{X}}$, wherein X is an integer; and

ketones or aldehydes defined by the formulas:

$HOCR^1R^2(CR^3R^4)_nCOR^5$;

$HOCR^1R^2(CR^3R^4)_nCHO$;

$R^1CO(CR^2R^3)_nCOOR^4$; and

$R^1CO(CR^2R^3)_nCOR^4$;

wherein n is 0 or an integer; and

wherein R^1 , R^2 , R^3 , R^4 , R^5 and R^6 are each independently selected from the group consisting of hydrocarbon radicals, halo- and hydroxy-substituted radicals, hydroxy radicals, halogen radicals, nitrogen-substituted radicals, oxygen-substituted radicals, and hydrogen atoms.

7. The method of claim 1 comprising

a) providing a plant biomass containing a PHA;

b) pre-processing the plant biomass to obtain a mixture containing PHA, oil and plant meal;

c) extracting oil from the mixture with a first solvent in which the oil is soluble and in which the PHA is not highly soluble, to obtain a residual meal mixture comprising PHA; and

d) extracting the residual meal mixture obtained in step c) with a second solvent in which the PHA is soluble, to separate PHA from the biomass.

8. The method of claim 1 comprising

a) providing a plant biomass containing a PHA;

- b) pre-processing the plant biomass to produce a mixture containing PHA, oil and plant meal;
- c) extracting oil from the mixture with a first solvent in which the oil is soluble and in which the PHA is not highly soluble, to obtain a residual meal mixture comprising PHA;
- d) treating the residual meal mixture comprising PHA, obtained in step c), with at least one chemical or biochemical agent, to chemically derivatize the PHA; and
- e) separating derivatized PHA from the residual meal mixture obtained in step d).

9. The method of claim 7 or 8 wherein step b) comprises pre-processing the plant biomass using one or more processes selected from the group consisting of drying, dehulling, cleaning, ageing, cleaning, weighing, cracking, flaking, pressing, rolling, grinding, cooking, crushing, settling and filtering.

10. The method of claim 7 or 8 wherein the first solvent comprises a hydrocarbon.

11. The method of claim 10 wherein the first solvent comprises a hydrocarbon selected from the group consisting of propane, butane, pentane, hexane, heptane, octane, nonane and decane.

12. The method of claim 7 wherein the second solvent is selected from the group consisting of a chlorinated organic solvent, an alkyl carbonate, an alcohol, and a hydroxyacid and mixtures thereof.

13. The method of claim 12 wherein the second solvent comprises a chloronated organic solvent selected from the group consisting of chloroform, methylene chloride, dichloroethane, trichloroethane, tetrachloroethane and dichloroacetate.

14. The method of claim 7 wherein the second solvent comprises a composition selected from the group consisting of a propylene carbonate,

ethylene carbonate, trifluoroethanol, acetic anhydride, acetic acid, dimethylformamide, ethylacetoacetate, triolein, toluene, dioxan, tetrahydrofuran, diethylether and pyridine.

15. The method of claim 8 wherein, in step d), the residual meal mixture comprising PHA is treated with at least one chemical agent selected from the group consisting of an acid, a base, a detergent, an oxidizing agent, a chelating agent, a reducing agent, a nucleophilic reagent, an electrophilic reagent, a metal ion, an aqueous solution and an organic solution.

16. The method of claim 8 wherein the PHA is derivatized by a chemical transformation selected from the group consisting of an esterification, transesterification, hydrolysis, saponification, aminolysis, thiolysis, etherification, silylation, addition, elimination, rearrangement, and a condensation.

17. The method of claim 8 wherein the biochemical agent is an enzyme.

18. The method of claim 17 wherein the enzyme is selected from the group consisting of a depolymerase, protease, nuclease, lipase, phosphorylase and a glycosidase.

19. The method of claim 8 wherein, in step e), the derivatized PHA is separated by a physical process selected from the group consisting of distillation, extraction, centrifugation, filtration, and chromatography.

20. The method of claim 7 or 8 wherein the first and second solvents each have boiling points between 30°C and 250°C.

21. The method of claim 7 or 8 further comprising using a third solvent to precipitate the separated PHA.

22. The method of claim 1 wherein the biomass is derived from a plant source selected from the group consisting of soybean, cotton, coconuts, groundnuts, rapeseed, sunflower seed, olive, palm, sesame seed, linseed, castor, safflower seed, tobacco and potato.

23. The method of claim 4 wherein the separated PHA includes one or more of the same or different units selected from the group consisting of hydroxybutyrate, hydroxyvalerate, hydroxyhexanoate, hydroxyheptanoate, hydroxyoctanoate, hydroxynonanoate, and hydroxydecanoate.

24. The method of claim 1 wherein the separated PHA is selected from the group consisting of 3-hydroxybutyric acid, crotonic acid and alkyl esters thereof.

25. The method of claim 2 wherein the plant biomass is derived from a plant containing a heterologous PHA synthase gene derived from a microorganism selected from the group consisting of *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Chromatium*, *Flavobacterium*, *Halobacterium*, *Pseudomonads*, *Nocardia*, *Rhodococcus*, *Thiocystis*, *Streptomyces*, *Streptococcus* and *Zoogloea*.

26. A method for separating a polyhydroxyalkanoate ("PHA") from a plant biomass comprising plant oil, the method comprising extracting a plant biomass with a first solvent, in which the oil and PHA are soluble, to essentially remove oil and PHA from the biomass, and then separating PHA from the oil.

27. The method of claim 26 wherein the plant biomass is derived from a transgenic oil crop plant.

28. The method of claim 26 wherein the method comprises

- a) providing a plant biomass containing a PHA;
- b) pre-processing the plant biomass to obtain a mixture of PHA, oil and plant meal;
- c) extracting oil and PHA from the mixture with a first solvent in which the oil and the PHA are soluble and in which the meal is not highly soluble; and
- d) separating the PHA from the oil.

29. The method of claim 28 wherein the PHA is separated from the oil in step d) by treating the PHA-oil mixture with a chemical or biochemical agent, thereby to chemically derivatize the PHA, and separating derivatized PHA from the oil.

30. The method of claim 28 wherein the solvent is selected from the group consisting of a chloronated organic solvent, an alkylcarbonate, an alcohol, a hydroxyacid and a hydrocarbon, and mixtures thereof.

31. The method of claim 28 wherein the solvent is selected from the group consisting of hexane, trifluoroethanol, acetic anhydride, dimethylformamide, ethylacetacetate, triolein, acetic acid, toluene, dioxane, tetrahydrofuran, diethylether and pyridine.

32. The method of claim 29 wherein the biochemical agent is an enzyme selected from the group consisting of a PHA depolymerase, protease, lipase, esterase, hydratase and a phosphorylase.

33. The method of claim 7, 8, 28 or 29 wherein the separated PHA includes a functional group selected from the group consisting of esters, amides, thioesters, acids, ethers, esters, unsaturated compounds, diols, ketones and aldehydes.

34. The method of claim 7, 8, 28 or 29 wherein the separated PHA includes one or more units selected from the group consisting of a 3-hydroxyacid, a 4-hydroxyacid and a 5-hydroxyacid.

35. The method of claim 7 or 28 wherein the separated PHA is separated in an organic solvent; and wherein the method further comprises:

emulsifying the PHA in the organic solvent in an aqueous solution containing a surfactant, thereby to form a PHA latex.

METHODS FOR ISOLATING
POLYHYDROXYALKANOATES FROM PLANTS

Abstract

Methods are provided for separating polyhydroxyalkanoates ("PHAs") from plants, such as transgenic oil crop plants. The methods advantageously permit both the oil and the PHAs to be recovered from the plant biomass. To isolate the PHAs, in one embodiment, a biomass derived from an oil crop plant is pre-processed, for example by grinding, crushing or rolling. The oil then is extracted from the biomass with a first solvent in which the oil is soluble and in which the PHAs are not highly soluble to remove the oil. The biomass then can be extracted with a second solvent in which the PHA is soluble, to separate the PHA from the biomass. Alternatively, the PHA-containing biomass is treated with a chemical or biochemical agent, such as an enzyme, to chemically transform the PHA into a PHA derivative. The PHA derivative then is separated from the mixture using, for example, a physical separation process such as distillation, extraction or chromatography. Advantageously, using the method, the plant oils, the PHAs and PHA derivatives can be recovered and purified on a large scale from oil containing plants such as transgenic oil crop plants.

Continuation of U.S.S.N. 08/548,640
MBX 512 236

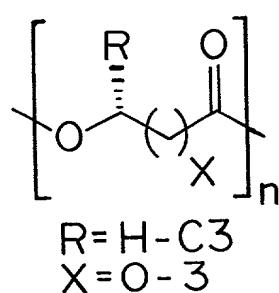


FIG. 1a

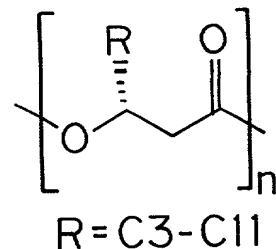


FIG. 1b

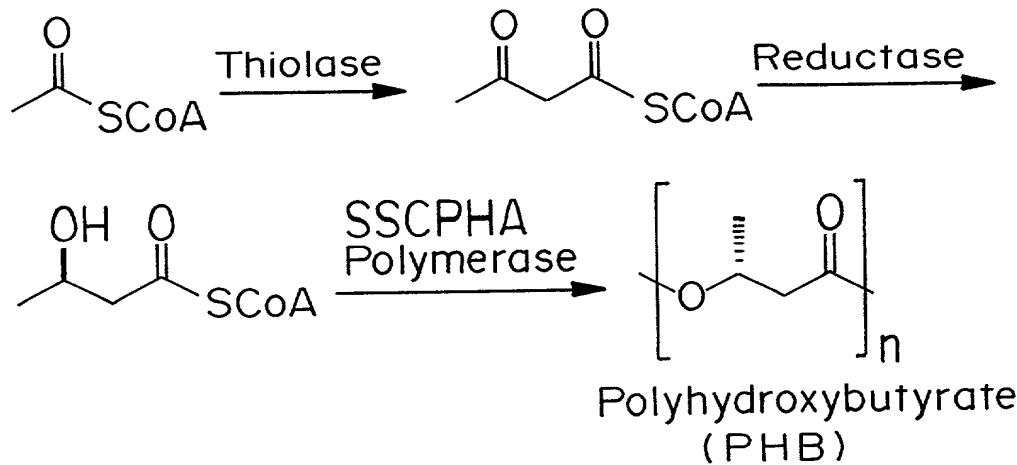


FIG. 2a

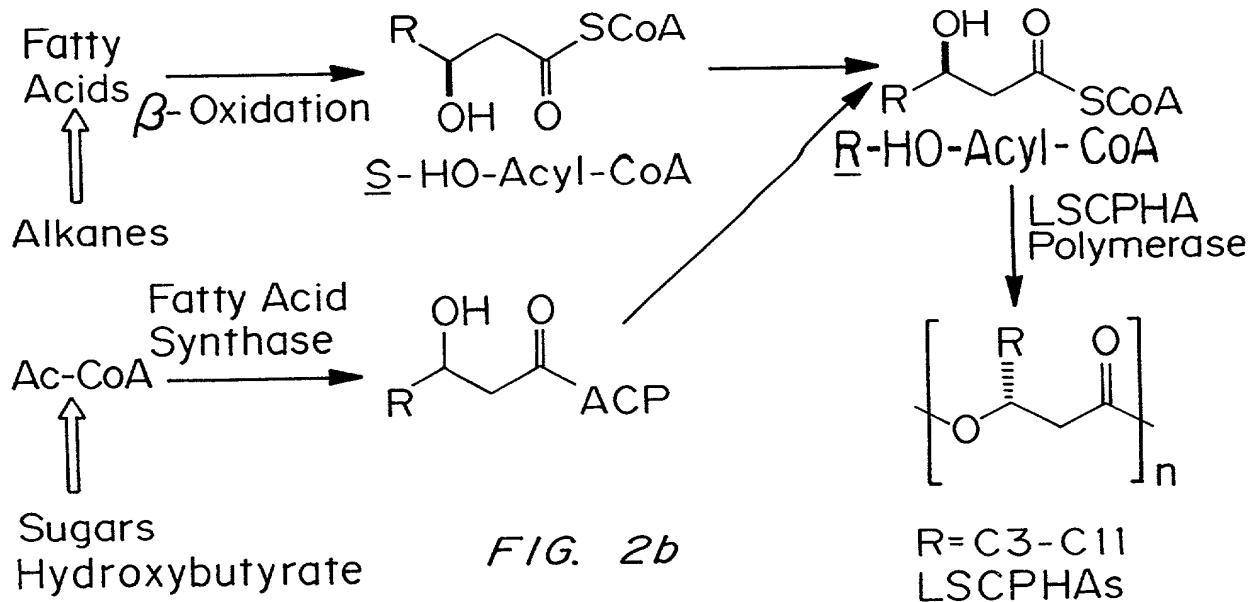
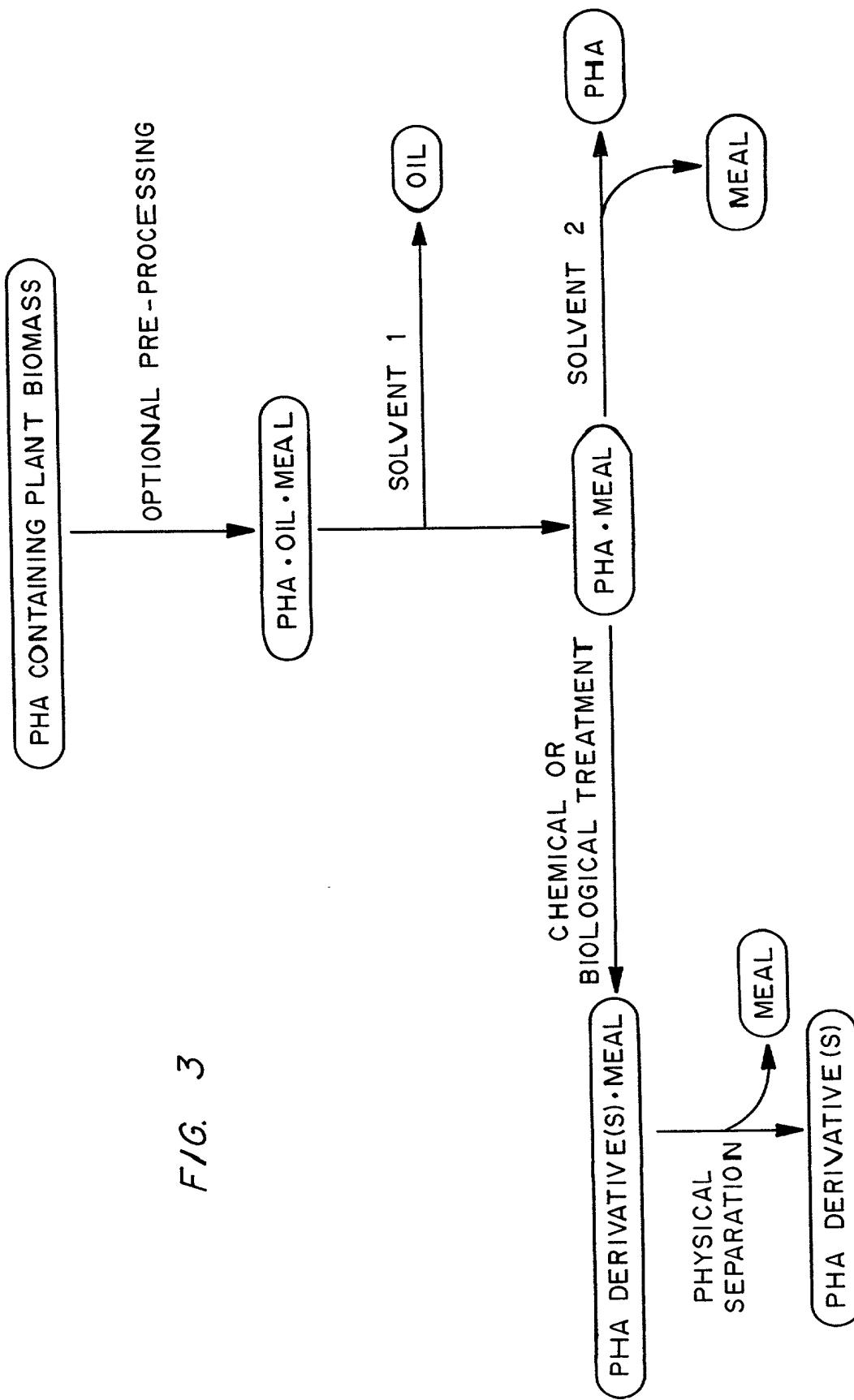


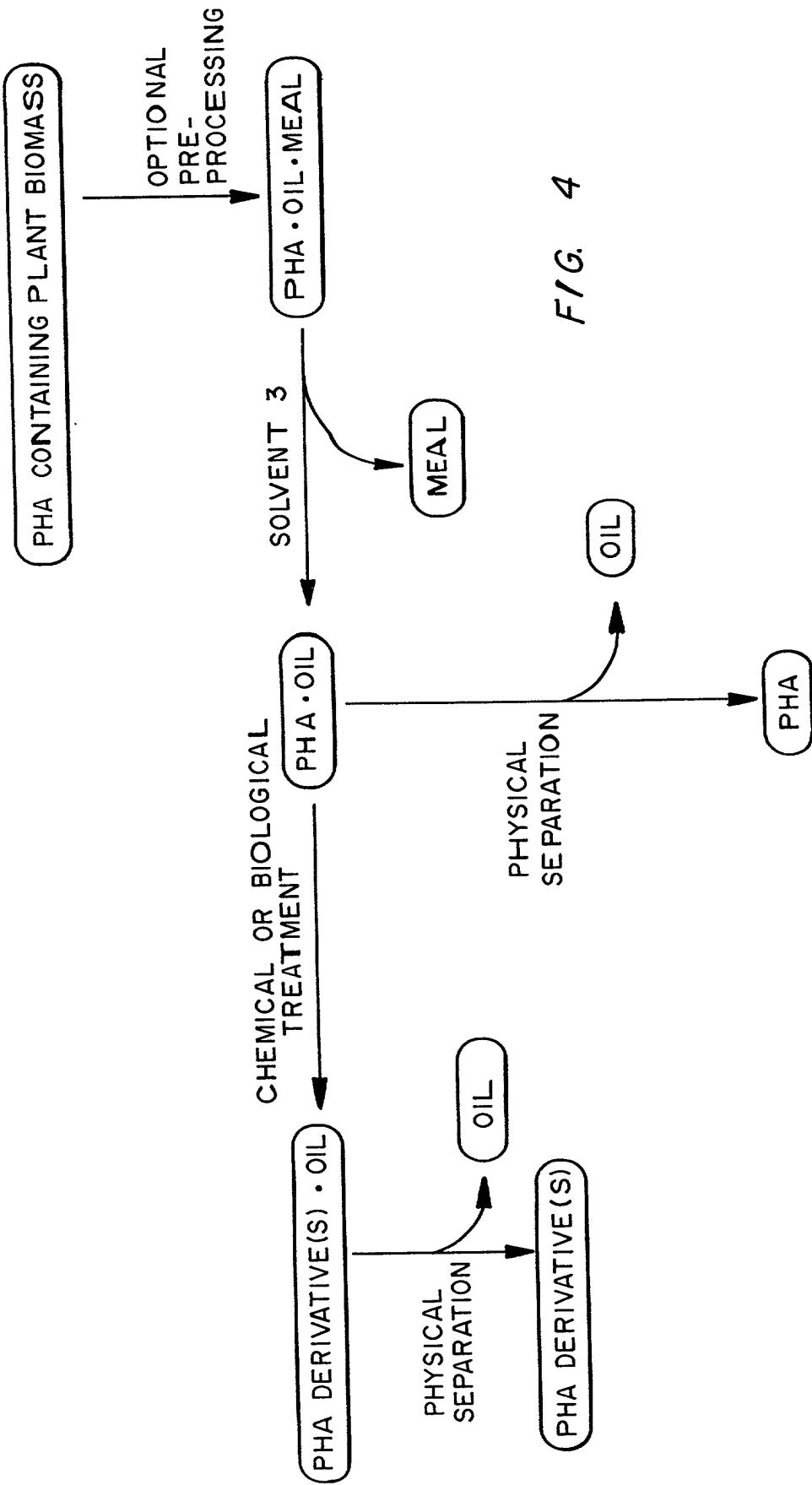
FIG. 2b

$\text{R} = \text{C3-C11}$
LSCPHAs

Continuation of U.S.S.N. 081542,240
MBX 012 2015



Continuation of Serial No 08/348,240
MBX 012 cont'



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: David P. Martin, Oliver P. Peoples, and Simon F. Williams

Serial No.: Continuation of 08/548,840 Group Art Unit:

Filed: June 12, 2000 Examiner:

For: METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS

Assistant Commissioner for Patents
Washington, D.C. 20231

ASSOCIATE POWER OF ATTORNEY UNDER 37 C.F.R. § 1.34

Sir:

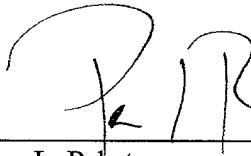
Please recognize as Associate Patent Attorneys and Patent Agent in this case:

Robert A. Hodges Reg. No. 41,074
Kevin W. King Reg. No. 42,737

Please continue to direct all communication to Patrea L. Pabst at the following address:

**Patrea L. Pabst
ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450
(404) 873-8794; 873-8795 (fax)**

Date: June 12, 2000



Patrea L. Pabst
Reg. No. 31,284

ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450
(404) 873-8794
(404) 873-8595 (fax)

792532v1

MBX 012 CON
20061/18

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS FOR ISOLATING POLYHYDROXYALKANOATES FROM PLANTS

the specification of which (check one)

is attached hereto

was filed on _____
as application Serial No. _____

and was amended on: _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)	Priority <u>Claimed</u>
_____ (Number)	_____ (Country) _____ (Day/Month/Year Filed)
_____ (Number)	_____ (Country) _____ (Day/Month/Year Filed)
_____ (Number)	_____ (Country) _____ (Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

_____ (Application Serial No.)	_____ (Filing Date)	_____ Status (patented, pending, abandoned)
-----------------------------------	------------------------	---

"Methods for Isolating Polyhydroxyalkanoates from Plants
By:David P. Martin, et al.
Filed: Herewith
DECLARATION

As named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Patrea L. Pabst	31,284
Madeline I. Johnston	36,174
Dolly A. Vance	39,054

Send Correspondence to: Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY
2800 One Atlantic Center
1201 W. Peachtree Street
Atlanta, GA 30309-3450

Direct telephone calls to: Patrea L. Pabst (404)873-8794

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor David P. Martin

Inventor's signature [Signature] Date 10/24/95

Residence 82 Hibbert Street, Arlington, Massachusetts 02174

Citizenship U.S.A.

Post Office Address Same as above

Full name of second joint inventor (if any) Oliver P. Peoples

Inventor's signature [Signature] Date 10/24/95

Residence 27 Radcliffe Road, Arlington, Massachusetts 02174

Citizenship Scotland

Post Office Address Same as above

"Methods for Isolating Polyhydroxyalkanoates from Plants

By: David P. Martin, et al.

Filed: Herewith

DECLARATION

Full name of third joint inventor (if any) Simon F. Williams

Inventor's signature Simon F. Williams

Date 10/24/95

Residence 26 Deerfield Road, Sherborn, Massachusetts 01770

Citizenship Great Britain

Post Office Address Same as above